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The Human Cytomegalovirus UL55 (gB) and UL75 (gH) Glycoprotein Ligands Initiate the Rapid Activation of Sp1 and NF-κB during Infection

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The cellular transcription factors Sp1 and NF-κB were upregulated shortly after the binding of purified live or UV-inactivated human cytomegalovirus (HCMV) to the cell surface. The rapid time frame of transcription factor induction is similar to that seen in other systems in which cellular factors are induced following receptor-ligand engagement. This similarity suggested that a cellular receptor-viral ligand interaction might be involved in Sp1 and NF-κB activation during the earliest stages of HCMV infection. To focus on the possible role viral ligands play in initiating cellular events following infection, we first used purified viral membrane extracts to demonstrate that constituents on the membrane are responsible for cellular activation. Additionally, these studies showed, through the use of neutralizing antibodies, that the viral membrane mediators of this activation are the major envelope glycoproteins gB (UL55) and gH (UL75). To confirm these results, neutralizing anti-gB and -gH antibodies were used to block the interactions of these glycoproteins on whole purified virus with their cell surface receptors. In so doing, we found that Sp1 and NF-κB induction was inhibited. Lastly, through the use of purified viral gB protein and an anti-idiotypic antibody that mimics the image of the viral gH protein, it was found that the engagement of individual viral ligands with their appropriate cell surface receptors was sufficient to activate cellular Sp1 and NF-κB. These results support our hypothesis that HCMV glycoproteins mediate an initial signal transduction pathway which leads to the upregulation of host cell transcription factors and suggests a model wherein the orderly sequence of virus-mediated changes in cellular activation initiates with viral binding via envelope glycoproteins to the cognate cellular receptor(s).

One of the hallmarks of human cytomegalovirus (HCMV) infection is the massive induction and dysregulation of normally tightly regulated cellular transcription factors, including NF-κB, Sp1, and others (1, 8, 9, 28, 33, 62, 78, 79), and because HCMV is associated with a wide range of clinical symptoms in which cellular activation occurs (33), it is possible that this virus-mediated cellular event is linked to viral pathogenicity. Previously, it was demonstrated that infection resulted in a rapid induction of nuclear NF-κB and increased steady-state message levels for the two classical NF-κB subunits, p50 and p65 (9, 49, 78). In addition, it was shown that the promoters for these two subunits are transactivated during infection and that the major viral immediate-early (IE) gene products play a role in this transactivation (78). There was also an upregulation of the usually constitutively expressed Sp1 during HCMV infection (79). The observed HCMV-induced increase in Sp1 levels may be the cause of the upregulation of p65 expression because the Sp1 sites present in the p65 promoter were shown to be critical for transactivation of the p65 promoter during infection (79). The importance of the induction of these host cell factors stems from their critical role during infection in the regulation of the major IE promoter (MIEP) and the regulation of the IE genes and, consequently, the entire gene cascade (13, 28, 62, 66). Specifically, NF-κB binds to and is essential for transactivation of the MIEP (13, 62), while Sp1, although studied in less detail, can also significantly transactivate the MIEP (77). The essential role for NF-κB in the viral life cycle is further underscored by the fact that no other stimulus has been found to induce the normally constitutively expressed p65 gene product (49, 78). In addition, it is intriguing to consider that the proto-oncogenes (c-*fos*, c-*jun*, and c-*myc*) which are rapidly induced following HCMV infection and are suggested to be one of the driving forces behind the stimulation of cellular DNA synthesis and proliferation during infection (8) have functional or potentially functional κB (26, 27, 45) and/or Sp1 (12, 46, 52, 70) binding sites in their promoters. Therefore, because of the coupling of virus-induced transcription factors to the regulation of the viral life cycle, we have focused on the mechanisms involved in the regulation of two critical players, NF-κB and Sp1, during HCMV infection.

NF-κB or classical NF-κB is a heterodimer composed of two members of the *rel* family of transcription factors: a 50-kDa subunit (p50 or NF-κB1) and a 65-kDa subunit (p65 or RelA) (reviewed in references 5, 64, and 69). In addition to classical NF-κB, other *rel* family transcriptional regulators exist (5, 64,

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69). Under nonstimulatory conditions, NF-κB is stored in the cytosol primarily complexed to an inhibitor termed IκBα (3–5, 30, 64, 69). During stimulation, NF-κB is rapidly released from its inhibitor, allowing its mobilization to the nucleus (5, 25, 64, 69). Sp1 is a member of a family of transcription factors known to bind to specific GC-rich elements (23, 24, 36, 47). The mechanisms involved in the control of Sp1 activity remains unknown but may involve an elusive inhibitory molecule (12, 70) which possibly binds free Sp1, blocking its transcriptional abilities. pRb and other Sp family members can also play a role in the regulation of Sp1 function (12, 46, 51, 52, 70, 71).

Previous investigations of the viral regulation of cellular transcription factors in HCMV infection have demonstrated that at least two steps or tiers are involved. First, through an unknown mechanism, there is an initial upregulation or mobilization of factors which occurs within minutes after virus-cell interaction, is protein synthesis independent (9, 78), and probably results from the release of cytosolic stores through the disruption of inhibitory complexes (for NF-κB, this would involve the NF-κB/IκB complex [49, 73, 78], and for Sp1, this might involve the several described inhibitors [12, 70]). Second, there is a synthesis of new molecules in a protein synthesis-dependent step via transactivation of the p105/p50 and p65 promoters (new NF-κB molecules) and also, possibly, the Sp1 promoter (new Sp1 molecules) beginning at early times after infection and continuing through the course of infection (78, 79). This second tier of transcription factor induction has been documented to be mediated by a combination of cellular factors and viral IE products (78, 79).

The steps involved in the initial tier of regulation are unknown. However, because the rapid kinetics of NF-κB activation by HCMV infection (9, 78) is reminiscent of the NF-κB induction following cytokine and/or mitogen treatment, a known receptor-ligand-mediated interaction (5, 64, 69), we hypothesized that a similar event, a virus-regulated receptor-ligand interaction, might exist following HCMV infection. This hypothesis is supported by evidence that upon HCMV infection, many of the IE events that take place, including Ca^{2+} flux, phospholipid turnover, induction of second messengers, and induction of the proto-oncogenes, are similar to events that occur following known receptor-ligand interactions in other systems (for reviews, see references 1, 2, 7, and 76). The possibility of a viral ligand-mediated IE signaling event occurring during HCMV infection is also supported by studies documenting that many viruses initiate infection by binding to what have been called cellular "adhesion" receptors (6, 20, 21, 48, 53, 56, 65, 68, 72), many of which are considered "activational" receptors (76).

If a receptor-ligand interaction were involved in this early viral step of cellular activation, the most likely ligand candidates would be the viral envelope glycoproteins (35, 58). Based on homology to herpes simplex virus, HCMV encodes two major glycoproteins, gB (UL55, gcI [18, 58]) and gH (UL75, gcIII [19, 57]). The believed role for these viral glycoproteins is in virus attachment to the cell surface and in the ensuing virus fusion and entry (15, 16, 29, 38, 54, 55, 65). gB is a posttranslationally processed, disulfide-linked heterodimer found on the viral envelope (10, 18, 29, 58) that is important in virion penetration and fusion (54). In addition, gB has been shown to bind to heparan sulfate proteoglycans and annexin II on the cell surface (15, 17, 75). The second major envelope glycoprotein, gH (19, 35, 57), is complexed to gL (UL115 [37], of which little is known), has been shown to bind to a ubiquitously expressed 92.5-kDa cellular phosphoprotein found on most cell types (39, 42), and is also important in virus fusion (38).

Our hypothesis for a potential role of HCMV glycoproteins in mediating a signal transduction pathway is further supported by evidence that the engagement of the 92.5-kDa cellular gH receptor by an anti-idiotypic antibody, which mimics HCMV gH, results in a rapid increase in cytoplasmic and nuclear $[Ca^{2+}]$ (40), as well as an increase in phosphorylation of the 92.5-kDa receptor (41). To address the nature of the first tier of regulation, we focused on the initial cellular events that occur following virus binding. We showed that present on the viral envelope are the factors necessary for transcription factor induction and that viral glycoproteins gB and gH can upregulate Sp1 and NF-κB levels. These results provide evidence that the receptor-ligand engagement that occurs during viral binding generates a sufficient signal to initiate the cellular signal transduction pathway and suggests that upon binding to the cell, HCMV attempts to mimic a cellular activation process to possibly set the stage for viral gene expression.

MATERIALS AND METHODS

Cell culture and virus. Methods for HCMV Towne strain (passages 42 to 43) culture in human embryonic lung (HEL) fibroblasts have been described previously (31, 78). Prior to infection, HEL fibroblasts were subcultured in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum (Hyclone, Logan, Utah) at 37°C in a 5% CO_2 incubator. Cells were infected with only gradient-purified virus at a multiplicity of infection of 2 to 3 and incubated at 37°C in Eagle's MEM supplemented with 4% heat-inactivated fetal bovine serum (Hyclone) in a 5% CO_2 incubator for the various times stated in Result (from mock infection [uninfected or untreated] through 4 h postinfection). HCMV was gradient purified and used to infect cells as previously described (32). UV-inactivated virus was prepared as previously described (8) and was used in the same manner as live virus. The UV-inactivated virus did not replicate or produce any detectable levels of IE gene products.

Purification of viral membrane extracts. Gradient-purified virus was incubated in a lysis buffer (0.5% Nonidet P-40 [NP-40], 0.01 M Tris-HCl [pH 8.0], 0.15 M NaCl, 0.025% NaN_3) for 15 min at 4°C and then carefully layered onto a 30% sucrose cushion. The samples were then spun at 200,000 × *g* for 90 min at 25°C. The membrane fraction sitting above the sucrose cushion was collected and extensively dialyzed to phosphate-buffered saline (PBS) and then to Eagle's MEM and finally stored at −70°C until use. Contamination of the membrane with capsid components was checked by Western blot analysis. Equal-protein amounts of the purified membrane fraction and gradient-purified virus were used in the selected experiments.

gB plasmids, expression, and purification. The HCMV gB gene was amplified by using *Taq* polymerase from the genomic DNA of HCMV (Towne strain) by PCR with primers 5′-TGGATTTGGCAAGCTTCGAACATGGA-3′ and 5′-GACACGGCCAAAGCTTGTCATGACTC-3′. The gB sequence was confirmed by DNA sequencing analysis. Plasmid pcGB3 was constructed by digestion of the amplified gene with *Hin*dIII (Promega, Madison, Wis.) and inserted into plasmid pcDNA3 (Invitrogen, Portland, Oreg.). 293T cells were then transfected with the pcGB3 vector by the calcium phosphate method. Cells were then cloned and isolated by limiting dilution and examined by immunofluorescent staining with anti-HCMV gB-specific monoclonal antibodies (MCMVA98 [34] and 13-127-100 [Advanced Biotechnologies Inc., Columbia, Md.]). gB^+ cells were grown in Eagle's MEM supplemented with 10% fetal bovine serum (Hyclone) at 37°C in a 5% CO_2 incubator, collected, and lysed in a lysis buffer (10 mM Tris-HCl (pH 7.5) 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride [PMSF; Sigma, St. Louis, Mo.], 1% NP-40) for 1 h at 4°C. After clarification by centrifugation, the lysate was reacted with lentil (*Lens culinaris*) lectin-Sepharose 4B beads (Sigma) at 25°C for 1 h. The incubated product was then washed five times in a washing buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA, 1 mM PMSF, 0.5% NP-40), and the attached protein was eluted with 0.5 M α-methyl-D-mannopyranoside (Aldrich Scientific, Milwaukee, Wis.). The eluted protein was next electrophoresed on a 5% nondenaturing polyacrylamide gel, and the gB fragment was cut out (the fragment position was identified by Western blot analysis with specific anti-gB monoclonal antibodies (MCMVA98 and 13-127-100) and by Coomassie blue (Sigma) staining or silver staining (BioRad Kit; BioRad, Richmond, Calif.). The gB protein was then electroeluted and stored at 4°C until use. Simultaneous with harvesting of the purified gB product, a control protein lysate was harvested from parental 293T cells by the protocol described above.

Antibodies and cellular activation. For experiments involving cellular activation with purified membrane fractions, cells were incubated with a protein amount of purified viral membrane fractions equal to that found in the purified virus used to infect cells in those experiments or the same amount of purified membrane fractions preincubated with 20 μg of protein G affinity-purified murine monoclonal anti-gB (15D8 [60]) and anti-gH (1G6 [61]) antibodies of the immunoglobulin G2A [IgG2A] isotype for 1 h at 4°C prior to use. Different concentrations of antibody and membrane fractions were also used (data not shown). For various experiments involving antibody blocking of the gradient-

purified virus, virus was preincubated with monoclonal anti-gB antibodies (4 and 20 μg of 15D8 or a 1:100 dilution of an ascites fluid preparation of CH177-3, CH432-1, and CH436-1 [54, 59], depending on the experiment), anti-gH antibody (4 and 20 μg of 1G6), both anti-gB (15D8) and -gH (1G6) antibodies, or a protein G affinity-purified murine monoclonal control antibody (4 and 20 μg of an anti-human prostate-specific antigen (PSA) antibody [control antibody] of the IgG2A isotype) for 1 h at 4°C. For neutralization with the 15D8 monoclonal antibody, 2% guinea pig complement (Accurate Scientific, Westbury, N.Y.) was added. Where appropriate, complement alone or complement plus the control antibody was used (data not shown). The neutralizing antibodies used in the experiments prevented virus-mediated cytopathic effects and viral gene expression in fibroblasts as confirmed by side-by-side experiments in which these variables were assessed (data not shown). In addition, 15 and 60 ng of purified viral gB or purified viral gB blocked with antibodies (5 μg of 15D8 and 1G6 or the control antibody) were used in some studies to stimulate cells. In other studies, 50 μg of the 4-3-5 affinity-purified murine anti-idiotypic IgM antibody (38, 43), which mimics the HCMV viral gH product and is thus specific for the 92.5-kDa cellular gH receptor (42), or an IgM control antibody (affinity-purified mouse IgM [Southern Biotechnology Associates, Birmingham, Ala.]) plus 10 μg of the appropriate cross-linking antibody (goat anti-mouse IgM antibody [Southern Biotechnology Associates]) was used to stimulate cells as previously described (40, 41). In all cases of cellular stimulation (purified virus, purified viral membrane extracts, purified gB, anti-idiotypic antibody, or the various control products), the cells were treated the same (washed identically, incubated in an equal volume of product and medium, incubated for the same length of time, and harvested in the same manner) to allow comparisons between groups. In all cases, mock samples were treated in a manner similar to that of the test samples (except that no virus, membrane extracts, etc. were added) and incubated for the maximal length of test sample stimulation. All experiments were repeated two to four times.

Nuclear extract isolation. Isolation of nuclear extracts has been described elsewhere (49, 78). Briefly, collected cell pellets were incubated for 4 min on ice with a cytoplasmic isolation buffer (10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES, pH 7.6], 60 mM KCl, 1 mM EDTA, 0.1% NP-40, 1 mM dithiothreitol, 1 mM PMSF [Sigma], 2 mM phenanthroline [Sigma], 250 μM dichloroisocoumarin [Sigma], 100 μM E-64 [Sigma], 10 μM pepstatin A [Sigma]). After collection by centrifugation, the nuclear pellets (the cytoplasmic extracts were not used in these experiments) were washed in a cytoplasmic isolation buffer without NP-40, spun, and then incubated for 10 min on ice with a nuclear isolation buffer (20 mM Tris-HCl [pH 8.0], 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF [Sigma], 25% glycerol, 2 mM phenanthroline [Sigma], 250 μM dichloroisocoumarin [Sigma], 100 μM E-64 [Sigma], 10 μM pepstatin A [Sigma]). Supernatants containing the nuclear extracts were collected and stored at −70°C.

Electrophoretic mobility shift assays (EMSAs). Collected nuclear extracts were incubated for 15 min in a binding buffer (10 mM Tris-HCl [pH 7.9], 50 mM NaCl, 0.5 mM EDTA, 10% glycerol, 1 mM dithiothreitol)–7.5 mM $MgCl_2$–0.1 μg of poly(dI-dC) with a ^{32}P-labeled wild-type (WT) GC box (5′-CCTTTTTAAG GGGCGGGGCTT-3′) or a mutant (MUT) GC box 5′-CCTTTTTAAGGTTC GGGGCTT-3′) double-stranded oligonucleotide probe for the experiments examining Sp1 activity and a major histocompatibility complex (MHC) WT κB binding site (5′-CCTTTTTTTTTGGGGATTCCCCA-3′) or a MUT κB binding site (5′-CCTTTTTTTTTGCGGCTTCCCGA-3′) double-stranded oligonucleotide probe for experiments examining NF-κB activity. The annealed double-stranded oligonucleotide probes with T overhangs and C ends were labeled by filling in the recessed 3′ ends of the oligonucleotide with [α-^{32}P]dATP (ICN, Irvine, Calif.) by using Klenow (Boehringer Mannheim, Indianapolis, Ind.), followed by a chase with cold dATP and dGTP, and then finally column purified with G-25 Sephadex (Boehringer Mannheim). The samples were electrophoresed on a 5% polyacrylamide gel, dried, and developed with intensifier screens at −70°C. Antibodies were used to supershift the specific complexes of interest by pretreating the extracts for 15 min to 1 h at 4°C with 1 μg of antibody prior to their addition to the binding buffer, $MgCl_2$, poly (dI-dC), and labeled probes. Commercial anti-Sp1 antibody and its appropriate blocking peptide (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.) and specific antibodies to p50 and p65 and their appropriate blocking peptides (a generous gift from Albert S. Baldwin, Jr. [14, 63]) were used in the supershift experiments. Blocking peptides were preincubated with the supershifting antibodies for 1 h at 4°C prior to their incubation with the various nuclear extracts.

Western blot analysis. Samples for Western blot analysis were prepared by boiling the samples in a sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer prior to use. The same amount of protein was added to each lane. Samples were subjected to sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis, and the proteins were transferred overnight to a nitrocellulose membrane (Immobilon-P; Millipore Corporation, Bedford, Mass.). The blots were then blocked in a 5% skim milk–0.1% Tween 20–PBS solution for 1 h, followed by incubation in a 2% skim milk–0.1% Tween 20–PBS solution for 1 h with the primary antibody, 1:400 αUL94 (74), 1:2,500 αgB (Advanced Biotechnologies Inc.), 1:1,000 αIE1-72 (6E1 [78]), or 1:500 αpp65/UL83. Next, the blots were washed, incubated with a 1:2,500 dilution of the appropriate horseradish peroxidase-conjugated secondary antibody in a 2% skim milk–0.1% Tween 20–PBS solution for 1 h (Sigma), washed again, and finally incubated with the developing agents and developed in accordance with the ECL protocol (Amersham Life Sciences, Arlington Heights, Ill.). For gB isolation, a PBS-skim milk-Tween 20 solution containing either a 1:1,000 anti-gB antibody (Advanced Biotechnologies Inc.) or a 1:100 dilution of serum from an HCMV-seropositive individual was used. The blots were washed and reacted for 1 h with a 1:10,000 dilution of the appropriate alkaline phosphatase-conjugated secondary antibody (Promega). The blots were developed by colorimetric assay using 3.3 mg of nitroblue tetrazolium (Promega) per ml and 1.6 mg of 5-bromo-4-chloro-3-indolylphosphate (Promega) per ml for 30 min at 25°C.

RESULTS

Viral infection upregulated cellular transcription factors Sp1 and NF-κB in a rapid manner. To better detail the IE changes in cellular transcription factors following HCMV binding to the cell surface, we examined by EMSA the rapid induction of nuclear Sp1 and NF-κB activity following the incubation of HEL fibroblasts with gradient-purified virus. The data shown in Fig. 1 demonstrated that both of these important cellular factors were rapidly upregulated after viral incubation (approximately fivefold induction of both transcription factors was seen by 60 min). An increase in Sp1 activity was seen by as early as 30 min following viral incubation (Fig. 1A), and an increase in NF-κB activity was seen by as early as 15 min following virus addition (Fig. 1B). In addition, to demonstrate the specificity of transcription factor induction, we also examined in the same nuclear extracts the induction of activated transcription factor–cyclic AMP response element-binding protein (ATF/CREB) DNA binding activity, which was reported to be induced only at late times of infection (44). The results of this experiment showed that ATF/CREB binding activity (data not shown) was not induced during IE times of infection like NF-κB and Sp1. Supershifting antibodies showed that the shifted bands were bona fide Sp1 (Fig. 1C, supershifted band marked with an asterisk) and NF-κB (p50/p65) and p50/p50 homodimers (Fig. 1D, supershifted bands marked with asterisks). The appropriate blocking peptides showed the specificity of the supershifting antibodies. Although Sp1 was the major component of the shifted complex (Fig. 1C), induction of a lesser concentration of other GC box-binding proteins was also detected during infection (the nature of these other GC box-binding proteins is unknown). Binding of the nuclear extracts to mutated GC box or MHC probes was also assayed (compare the binding of extracts to the WT GC BOX or WT MHC oligonucleotides to the binding to the MUT GC BOX and MUT MHC oligonucleotides), and the lack of specific bands confirmed the specificity of the identified bands. In addition, in competition experiments, the Sp1 bands were specifically competed with an excess of the unlabeled WT GC BOX oligonucleotide but not by an excess of the unlabeled MUT GC BOX oligonucleotide, and the p50/p65 and p50/p50 bands were specifically competed with an excess of unlabeled WT MHC oligonucleotides but not unlabeled MUT oligonucleotides (data not shown). UV-inactivated virus also rapidly induced both Sp1 and NF-κB (Fig. 1A and B, respectively), supporting the work of Boldogh et al., who demonstrated that gradient-purified and UV-inactivated viruses had identical effects on the cellular changes they examined (8, 9). This rapid induction of cellular factors following HCMV incubation is consistent with our hypothesis that a viral ligand-cellular receptor interaction is responsible for an early step of cellular activation.

Purified HCMV membrane extracts induced Sp1 and NF-κB DNA binding activity, and gB and gH antibodies blocked this induction. The rapid upregulation of transcription factors following HCMV infection suggests that viral membrane glycoproteins might play a role in the initial cellular activation. To better define the nature of this viral induction of Sp1 and

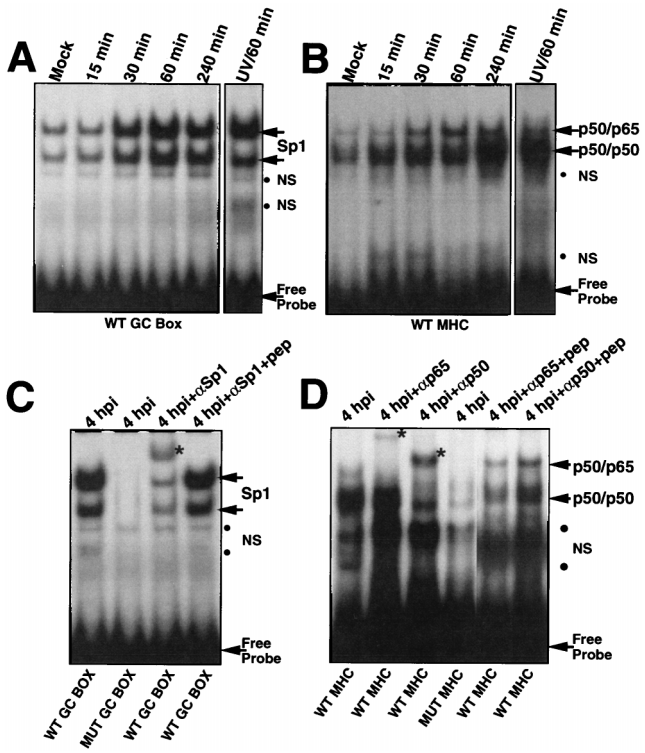

FIG. 1. HCMV infection results in rapid upregulation of Sp1 and NF-κB DNA binding activity. (A) EMSA of a time course of Sp1 DNA binding activity following infection with live or UV-inactivated, gradient-purified HCMV (multiplicity of infection, 2 to 3) of HEL fibroblasts. (B) Time course of NF-κB DNA binding following infection with purified live or UV-inactivated virus. (C) EMSA confirming the specificity of the induced Sp1 products via supershift experiments and binding of extracts to mutant probes. (D) EMSA confirming the specificity of the induced NF-κB products. Mock lanes contained uninfected fibroblasts treated in a manner identical to that of infected cells, except that no virus was added and they were incubated for the maximal length of sample stimulation. ATF/CREB DNA binding was not induced at IE times after infection (data not shown). The time course of infection represents the times of cell harvesting following virus addition. The binding of harvested HEL nuclear extracts to a consensus WT (5′-CCTTTTTAAGGGGCGGGGCTT-3′) or MUT (5′-CCTTTTAAGGTTCGGGGCTT-3′) GC BOX and a WT (5′-CCTTTTTTTTTGGGGATTCCCCA-3′) or MUT (5′-CCTTTTTTTTTGCGGCTTCCCGA-3′) MHC binding site was assessed. The specific (Sp1 and supershifted Sp1 [asterisks], p50/p65 and p50/p50 complexes, and supershifted p50 and p65 complexes [asterisks]) and nonspecific (NS) bands are labeled. Santa Cruz anti-Sp1 antibody (lane αSp1) and the appropriate blocking peptide (lane αSp1+pep) and specific anti-p50 and -p65 antibodies (lanes αp65 and αp50 [14, 63]) and their appropriate blocking peptides (lanes αp65+pep and αp50+pep) were used in the supershift experiments. In addition, competition experiments with unlabeled competitors were also done to show the specificity of the bound products (data not shown). hpi, hours postinfection.

NF-κB and to determine if a factor associated with the HCMV membrane envelope mediates the response, we enriched for viral envelope-membrane components and then used these products as cellular activators to test for transcription factor upregulation. Incubation of the purified membrane extract fractions with HEL fibroblasts for 60 min induced both Sp1 (Fig. 2A) and NF-κB (Fig. 2B) EMSA activities (compare the Mem lanes—incubation of cells with the purified membrane fractions— to the untreated Mock lanes), demonstrating that the signaling was mediated by a viral membrane constituent. The membrane-mediated induction of Sp1 and NF-κB activity could be blocked by preincubation (for 60 min) of the membrane samples with affinity-purified monoclonal anti-gB (15D8 [60]) and -gH (1G6 [61]) antibodies (compare the Mem lanes to the antibody-treated membrane fraction [Mem+αgBgH] lanes). Preincubation of the purified membrane extracts with an identical concentration of an isotype- and species-matched, nonspecific control antibody (Cont Ab lane) had no effect on the signaling capacity of these extracts. Examination of the membrane fractions by Western blot analysis confirmed the presence of the envelope glycoproteins and the purity of the

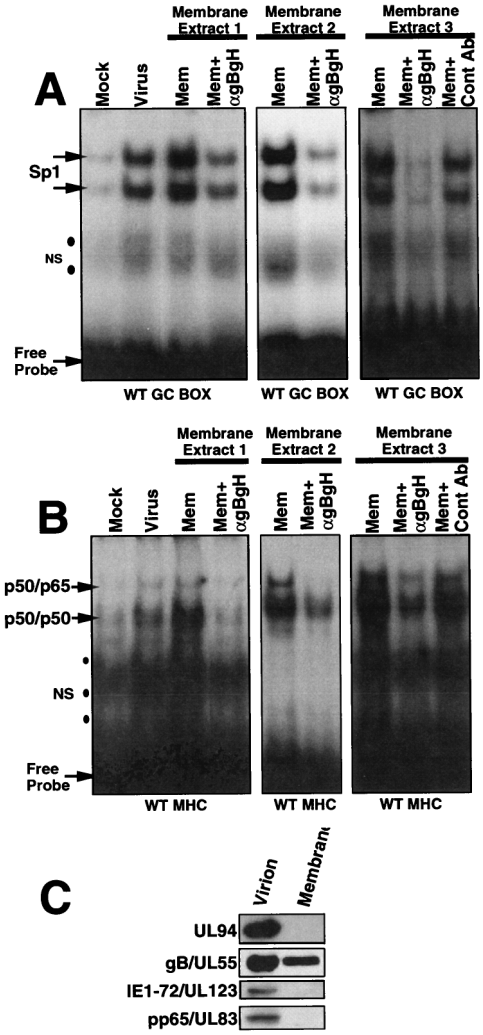

FIG. 2. Purified HCMV membrane fractions can modulate cellular transcription factor activity. (A) Changes in Sp1 DNA binding activity were examined in EMSAs by using lysates from HEL fibroblasts harvested following 60 min of incubation with either purified virus (Virus lane), purified viral envelope extracts (Mem lane; the same protein amount as was used with the purified virus), or purified envelope fractions preincubated for 60 min with affinity-purified anti-gB (15D8 [60]) and -gH (1G6 [61]) (Mem+αgBgH lane) antibodies or a species- and isotype-matched control antibody (Cont Ab [the anti-PSA antibody]). The Mock lane contained untreated cells handled the same way as treated cells except that no membrane extract was added and they were incubated for the same length of time as the test samples. (B) NF-κB DNA binding activity was analyzed in EMSAs by using lysates from fibroblasts harvested following incubation for 60 min with either purified virus (Virus lane), purified viral envelope fractions (Mem lane), or purified envelope fractions preincubated for 60 min with affinity-purified anti-gB and -gH (Mem+αgBgH lane) antibodies or the appropriate control antibody (Cont Ab [anti-PSA antibody). The Mock lane contained untreated cells handled the same way as treated cells, except that no membrane extract was added and they were incubated for the same length of time as the test samples. (C) The purity of the collected viral envelope fractions was demonstrated by Western blot analysis of the purified virion and viral membrane fractions. Samples were probed with antibodies specific for UL94 (74), gB (Advanced Biotechnologies Inc.), IE1-72 (78), or pp65/UL83. NS, nonspecific.

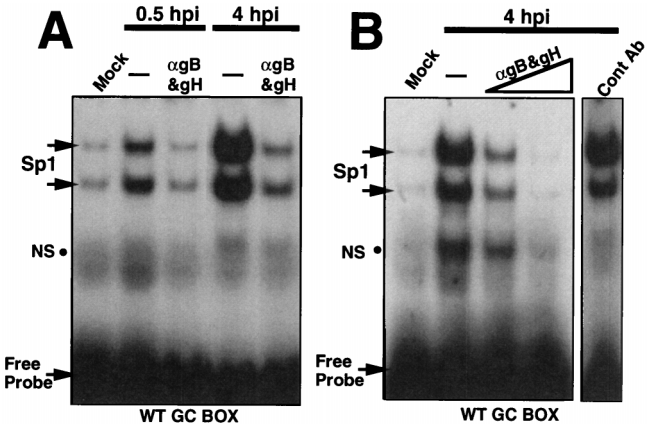

FIG. 3. Neutralizing antibodies to gB and gH block Sp1 induction. (A) EMSA of a time course of Sp1 DNA binding activity from HEL fibroblasts following infection with gradient-purified HCMV without or with pretreatment with neutralizing antibody (4 μg, preincubated for 60 min with anti-gB [15D8] and -gH [1G6] affinity-purified serum; lanes αgB&gH). (B) EMSA of Sp1 DNA binding following infection with purified virus or purified virus preincubated with 4 or 20 μg of anti-gB and -gH serum or 20 μg of an isotype- and species-matched control antibody (Cont Ab). See the Fig. 1 legend for a description of Mock lanes. The — lane contained cells infected with purified virus that were not preincubated with antiglycoprotein serum. NS, nonspecific; hpi, hours postinfection.

isolated fractions (Fig. 2C). UL94, a known capsid-associated HCMV gene product that is not found on the viral envelope (74); IE1-72, which we have previously documented to be present in the virion (74); and pp65/UL83 were all absent from our purified membrane extract fractions. The results of these experiments identify constituents of the viral membrane as the source of the early signaling event and suggest that viral glycoproteins gB and gH are the viral ligands responsible for this signaling process.

Antibody neutralization of viral glycoproteins gB and gH could prevent transcription factor upregulation. To confirm that viral membrane products mediated the induction of cellular factors, we further examined the role of the two primary membrane glycoproteins, gB and gH, by preincubating gradient-purified virus with a combination of specific neutralizing monoclonal antibodies (anti-gB [15D8] and -gH [1G6]) for 60 min prior to virus incubation with HEL fibroblasts. Preincubation of virus with these neutralizing antibodies blocked the increase in both Sp1 (Fig. 3) and NF-κB (Fig. 4) DNA binding activities seen with purified virus alone (compare the untreated/no-antibody (—) lanes to the antibody-treated (αgB&gH) lanes. Specifically, Fig. 3A and 4A demonstrated that preincubation of purified virus with anti-gB and -gH antibodies (4 μg of each) significantly blocked transcription factor upregulation at various early times postinfection (0.5 and 4 h postinfection). The antibody-mediated effect was dose dependent, because as the antibody concentration was increased from 4 to 20 μg, Sp1 and NF-κB induction was nearly completely blocked (Fig. 3B and 4B, respectively). Incubation of the gradient-purified virus with a similar concentration of a species- and isotype-matched control antibody had no effect on Sp1 (Fig. 3B) or NF-κB (Fig. 4B) signaling. Incubation of HEL fibroblasts with either antibody alone or antibody plus complement under the same conditions that were used for virus incubation or antibody-treated virus incubation had no effect on Sp1 or NF-κB levels or cell viability. In addition, in comparison experiments the neutralizing function of these antibodies was tested under similar conditions used to block transcription factor expression and was shown to prevent cytopathic effects and viral gene expression (results not shown).

Blocking of transcription factor induction by individual gB and gH antibodies. To broaden our examination of the ability of a different neutralizing antiserum to block the cellular signaling pathway, we preincubated purified virus with individual monoclonal anti-gB or gH antibodies. The 15D8 (αgB-15D8) and 1G6 (αgH-1G6) antibodies inhibited Sp1 (Fig. 5A) and NF-κB (Fig. 5B) activities when used alone. In addition, by using a panel of available anti-gB monoclonal antibodies (αgB-CH177-3, -CH432-1, and -CH436-1 [54, 59]) specific for different regions of the gB molecule, we also blocked transcription factor regulation. In contrast, the nonspecific isotype- and species-matched control anti-PSA antibody, when preincubated with virus, had no effect on the signaling cascade. Nonneutralizing gB antibodies (59) appeared to have no effect on the ability of purified HCMV to initiate the induction of nuclear Sp1 and NF-κB levels (76). These results support the involvement of the viral gB and gH glycoproteins in the early stage of the signaling cascade.

Purified HCMV gB induced Sp1 and NF-κB DNA binding upon cellular binding. The observations described above raise the possibility that the initial cellular induction by HCMV is due to viral ligand-receptor interactions. We next wanted to show that specific viral ligands are directly capable of inducing cellular transcription factors. Purified HCMV gB was incubated under conditions similar to those used for viral infection or treatment of cells with the membrane extracts. A 60-min incubation of purified HCMV gB with fibroblasts (lanes gB) induced Sp1 (Fig. 6A) and NF-κB (Fig. 6B) activities above those seen in untreated control cells with both amounts of gB used (15 and 60 ng). The flowthrough control, which was isolated from extracts from the parental 293T cells (no transfected gB) by the same procedure used to purify the gB, showed no ability to activate cellular transcription factors, thus demonstrating that the protocol used to purify gB did not itself have a mitogenic effect. Furthermore, the effects mediated by purified gB could be blocked by pretreatment with anti-gB antibody (15D8; compare lanes treated with antibody (αgB Ab lanes) to those without) but not by pretreatment with an iso-

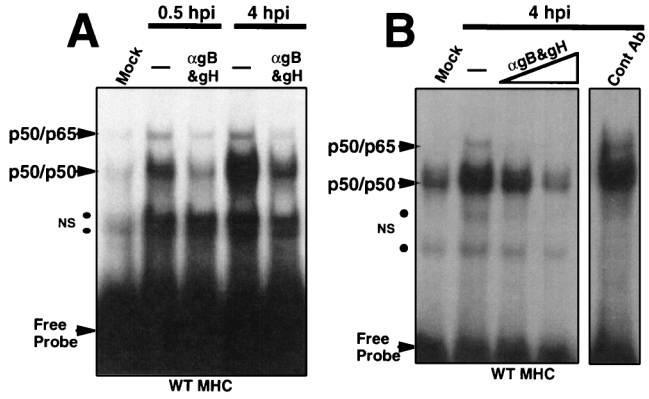

FIG. 4. Neutralizing antibodies to gB and gH also block NF-κB induction. (A) EMSA of a time course of NF-κB DNA binding activity following infection of fibroblasts with purified HCMV or neutralized HCMV (preincubated with 4 μg of affinity-purified anti-gB and -gH serum for 60 min; lanes αgB&gH). (B) EMSA of NF-κB DNA binding following infection with purified virus or purified virus preincubated with 4 and 20 μg of anti-gB and -gH serum or 20 μg of an isotype- and species-matched control monoclonal antibody (Cont Ab). See the Fig. 1 legend for a description of the Mock lane samples. The — lane contained cells infected with purified virus that were not preincubated with antiglycoprotein serum. NS, nonspecific; hpi, hours postinfection.

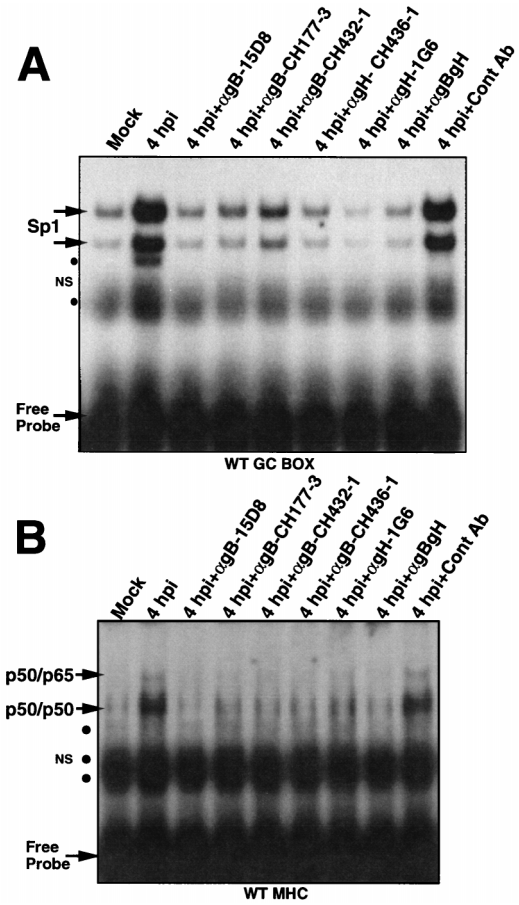

FIG. 5. Neutralization of purified HCMV with a panel of antibodies specific for gB and gH. (A) Measurement of Sp1 binding activity from nuclear lysates of harvested HEL fibroblasts incubated with either purified HCMV or purified HCMV preincubated for 60 min with anti-gB or -gH antibodies or an isotype-matched control antibody (Cont Ab). (B) Examination of NF-κB DNA binding activity of nuclear lysates harvested from fibroblasts incubated with either purified HCMV or purified HCMV preincubated with various antiglycoprotein (anti-gB or -gH) and control antibodies. The αgB-CH177-3, -CH432-1, and -CH436-1 antibodies (54, 59) were used as ascites fluid, while all of the other antibodies (αgB-15D8, αgH-1G6, and the control antibody) were used as affinity-purified antiserum. For a description of the Mock lanes, see the Fig. 1 legend. NS, nonspecific; hpi, hours postinfection.

type-matched control antibody (Cont Ab lanes). The significant increase in Sp1 and NF-κB activities following the incubation of cells with the purified gB product and the inhibition of these activities by anti-gB antibody demonstrated that the specific gB-receptor interaction could directly initiate signaling.

Interaction of an anti-idiotypic antibody that mimics gH with the gH cognate receptor also upregulated the signal transduction pathway. To show that the viral gH product also played a role in transcription factor induction, we used a well-characterized affinity-purified anti-idiotypic monoclonal (αId) antibody (38, 40–43) which antigenically mimics the viral gH product and binds to the same 92.5-kDa cell membrane protein as affinity-purified gH (42) to stimulate cells. The anti-idiotypic antibody (4-3-5) or the appropriate control (a nonspecific IgM) was preincubated for 60 min with a cross-linking (X-L) secondary antibody prior to 60 min of incubation with cells. The results of these experiments demonstrated that the engagement of the gH cellular receptor (lanes αId+X-L) could upregulate Sp1 (Fig. 7A) and NF-κB (Fig. 7B) DNA binding. The controls, i.e., the nonspecific IgM antibody plus the cross-linking antibody (αIgM Control+X-L) or the cross-linking antibody alone (X-L alone), could not induce transcription factors, thus demonstrating the specificity of the Sp1 and NF-κB upregulation via the gH anti-idiotypic antibodies.

DISCUSSION

The data demonstrates that a bona fide signal transduction event takes place following HCMV binding to the cell and that viral membrane constituents mediate this signaling event. More specifically, our results document that HCMV glycoproteins gB and gH are critical players in the induction of Sp1 and NF-κB during infection. Because of the essential role host cellular factors play during HCMV infection, these results suggest a model in which the receptor-ligand-mediated signaling event results in a burst of transcription factors that is vital for the initial induction of the important viral gene products which require cellular transcription factors for expression (9, 13, 28, 33, 62, 66). This data is supported by studies demonstrating that many viruses, including other members of the herpesvirus family, bind to known "activational" receptors (6, 20, 21, 48, 53, 56, 65, 68, 72). Although these studies neither directly examined nor demonstrated stimulation of cellular activation by viral binding, the conclusions drawn from these studies

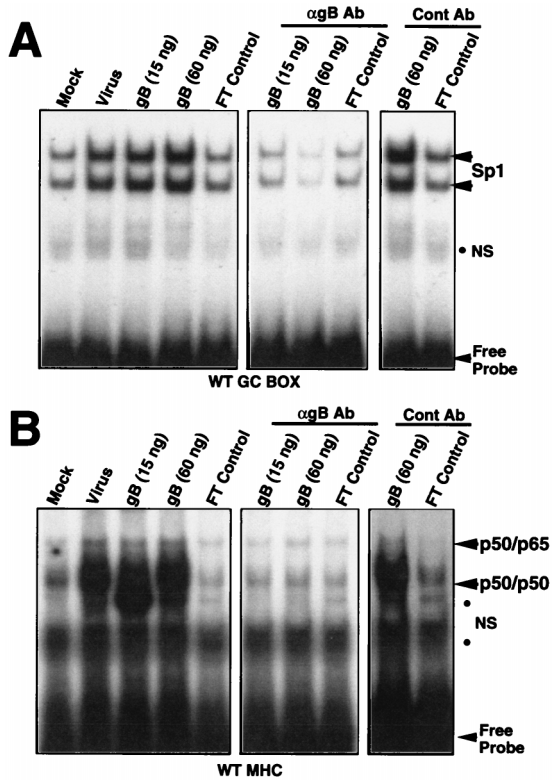

FIG. 6. Purified viral gB elicits a cellular response. (A) EMSA of Sp1 from nuclear lysates harvested from fibroblasts after 60 min of incubation with gradient-purified virus, purified gB (15 and 60 ng), or a flowthrough control lysate treated in the same manner as the purified gB (FT Control). In addition, in a replicate experiment, cells were treated with 5 μg of anti-gB (15D8 [αgB Ab]) or the appropriate control antibody (Cont Ab) for 60 min prior to incubation. (B) EMSA of NF-κB from nuclear lysates of fibroblasts treated with gradient-purified virus, purified gB, flowthrough control, or purified gB or flowthrough control lysate pretreated with anti-gB antibody or a control antibody. For a description of the Mock lanes samples, see the Fig. 1 legend. NS, nonspecific.

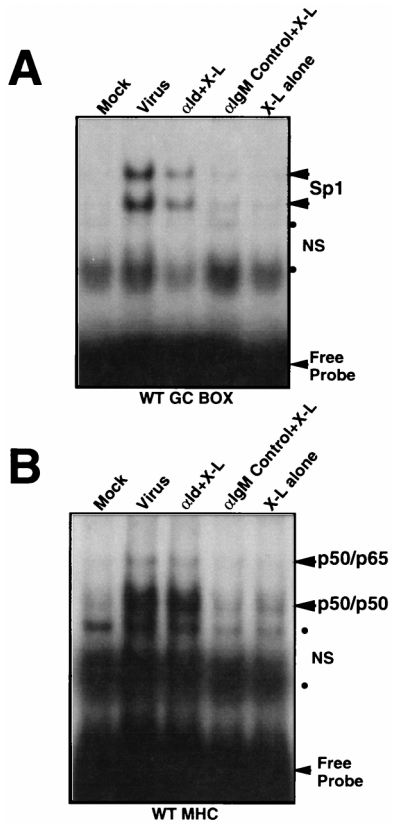

FIG. 7. Anti-idiotypic antibodies which mimic the HCMV gH glycoprotein can upregulate Sp1 and NF-κB EMSA activity. (A) EMSA of Sp1 binding activity from nuclear lysates following 60 min of incubation of fibroblasts with either purified virus, anti-idiotypic antibody (an IgM) plus the appropriate anti-IgM cross-linking antibody (αId+X-L), nonspecific IgM antiserum plus the cross-linking antibody (αIgM Control+X-L), or the cross-linking antibody alone (X-L alone). (B) EMSA examining NF-κB activity from nuclear lysates of fibroblasts treated with either purified virus, a specific anti-idiotypic antibody plus the appropriate cross-linking antibody (αId+X-L), nonspecific control antiserum plus the cross-linking antibody (αIgM Control+X-L), or the cross-linking antibody alone (X-L alone). For a description of the Mock lanes, see the Fig. 1 legend. NS, nonspecific.

tantalizingly hinted that this process might occur. Therefore, our present findings, along with those of past studies, support a hypothesis that viruses have evolved a common strategy to bind to a normally used activational molecule or receptor to facilitate not only viral entry but also cellular activation and, presumably, viral survival.

We have shown that there is a rapid increase in the levels of nuclear Sp1 and NF-κB DNA binding activity following the incubation of purified virus with fibroblasts, confirming the results of previous studies (9, 49, 62, 78, 79). In addition, the data suggested that the induction of these factors (Sp1 and NF-κB) was specific because ATF/CREB was not induced following viral binding (data not shown), supporting the results of Kerry et al. (44), who showed that ATF was upregulated only at late times of infection. The rapid induction of these cellular transcription factors is sufficient to account for the documented IE expression of the proto-oncogenes c-*fos*, c-*jun*, and c-*myc* (8), in which NF-κB and Sp1 have been documented to play a regulatory role (12, 26, 27, 45, 46, 52, 70). It could be argued that this IE signaling event was due to a mitogenic factor secreted by the infected cells from which we purified the virus. This possibility, however, was unlikely because when density gradient-purified virus was used, upregulation of transcription factors was still observed. In addition, UV-inactivated virus also induced cellular transcription factors. These findings are similar to those of work examining proto-oncogene expression, in which it was shown that density gradient-purified virus and inactivated virus had identical effects on proto-oncogene mRNA levels (8). Lastly, the antibody blocking studies supported this notion, because if a soluble mitogenic factor had been copurified with the virus, its effect would not have been specifically inhibited by the anti-HCMV antibodies.

Because the kinetics of the transcription factor induction was similar to that seen in systems in which receptor-ligand interactions are known to mediate the signaling event (5, 7, 64, 69, 76), we next wanted to determine that the viral products responsible for this rapid transcription factor induction are restricted to the viral membrane. Therefore, we enriched for the viral envelope fraction and used these products to stimulate cells. These envelope extract fractions stimulated Sp1 and NF-κB activity, supporting our hypothesis that ligands on the virus surface mediate the induction of Sp1 and NF-κB activity. Because anti-gB and -gH antibodies could block this stimulation and the isotype-matched control antibody could not, the data strongly hinted that it was these glycoproteins that were intimately involved with cellular regulation. Additionally, these results showed that the signaling was not due to a capsid- or tegument-associated product. Neutralizing monoclonal antibodies against gB and gH (together or alone) blocked the virus-mediated signal transduction event, providing additional support that it was, in fact, these viral ligands that mediated host cell transcription factor activation. Interestingly, nonneutralizing antibodies to gB (59) could not block the signaling cascade initiated by viral binding to the cell surface (77).

To further define the role the HCMV glycoproteins play in cellular signaling, purified HCMV gB and a well-characterized anti-idiotypic antibody (38, 40–43) that mimics the HCMV gH molecule were used. Purified gB directly stimulated host cell factors, whereas the control lysate could not. Furthermore, anti-gB antibodies specifically inhibited the purified gB-regulated transcription factor induction, while control antibodies did not, demonstrating that gB-receptor interaction could elicit the appropriate signal for the upregulation of Sp1 and NF-κB activity. The gH anti-idiotypic antibodies also induced these host cellular products, while the isotype control antibodies could not. Because these antibodies also generated an increase in $[Ca^{2+}]_i$ flux (40) and stimulated phosphorylation of the 92.5-kDa receptor (41), our results confirmed that gH receptor engagement could regulate a signaling event and, more importantly, showed that the engagement of the cellular gH 92.5-kDa receptor also provided the appropriate signal to upregulate Sp1 and NF-κB activity. Together, these studies define the HCMV gB and gH glycoproteins as active players in the signal transduction cascade and demonstrate that they can mediate a specific and sufficient signal to induce cellular activation. It is important to point out that there are other potential HCMV glycoproteins (10, 11, 19, 35, 37, 57, 58), and it is certainly possible that some of these gene products also play a role in vivo, particularly depending on the mechanism of virus transmission and/or the cell type infected.

The specific component(s) of the cellular pathway utilized by the virus following gB and/or gH engagement of their respective cellular receptors is unknown because little is known about the cellular receptors to which gB and gH bind and their intracellular signaling capacity. The rapid upregulation of nuclear NF-κB, however, probably results from the typical pathway of activation for this factor following receptor-ligand interaction, i.e., the mobilization to the nucleus of preformed

NF-κB from its cytosolic storage via the phosphorylation and proteasome-mediated degradation of IκBα or one of its other potential inhibitors (reviewed in references 5, 25, 64, and 69). Also probably involved is the Ras–Raf–mitogen-activated protein kinase signaling cascade, because these elements have been shown to be upstream of the phosphorylation and degradation of IκBα during receptor-ligand-mediated NF-κB stimulation (5, 25, 64, 69). The signaling pathway involved in the upregulation of Sp1 is unknown. It may involve some of the same components that are utilized in the regulation of NF-κB, i.e., the activation of certain regulatory kinases or phosphatases. Because these two products are differentially regulated in most documented cases, however, a divergent pathway is most likely used, perhaps one that targets the Sp1 regulatory molecule(s) (12, 46, 51, 52, 70, 71).

Our model describing the multiple levels of host cell transcription factor regulation can now be expanded to include our new results. As shown here, an initial induction of cellular factors occurs via gB and gH interaction with their appropriate cellular receptors. Following this receptor-ligand-mediated initial induction of cellular factors, we believe that there is an amplification of the signal by a virion-associated product(s) prior to induction of the IE genes. We have preliminary evidence that the HCMV capsid lacking an envelope upregulates host cell factors (77) and that a virion-associated kinase can phosphorylate IκBα (73). Perhaps, this product(s) is functionally analogous to the hepatitis B virus HBx protein (67), the human immunodeficiency virus Tat protein (22), or the human T-lymphotropic virus type I Tax_1 protein (50), at least in regard to its intracellular role in NF-κB activation. And lastly, as we have previously shown, viral IE genes can transactivate these cellular promoters and function along with the increased levels of cellular factors to maintain the high levels of these factors during the course of infection (76, 78). Overall, the data demonstrates that multiple pathways are utilized by HCMV for regulation of the vital transcription factors necessary for the maintenance and regulation of the viral life cycle (also presumably one of the underlying causes for the pathogenicity of the virus itself). In conclusion, we have defined a very early virus-regulated step that is responsible for the regulation of host cell factors involving the receptor-ligand interaction between the viral gB and gH glycoproteins and their cognate receptors.

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REFERENCES

1. **Albrecht, T., I. Boldogh, M. P. Fons, and T. Valyi-Nagy.** 1993. Activation of proto-oncogenes and cell activation signals in the initiation and progression of human cytomegalovirus infection, p. 384–411. *In* Y. Becker, G. Darai, and E. S. Huang (ed.), Molecular aspects of human cytomegalovirus diseases. Springer-Verlag KG, Berlin, Germany.
2. **Albrecht, T., M. P. Fons, I. Boldogh, S. AbuBaker, C. Z. Deng, and D. Millinoff.** 1991. Metabolic and cellular effects of human cytomegalovirus infection. Transplant. Proc. **23:**48–55.
3. **Baeuerle, P., and D. Baltimore.** 1989. A 65-kD subunit of active NF-κB is required for inhibition by IκB. Genes Dev. **3:**1689–1698.
4. **Baeuerle, P. A., and D. Baltimore.** 1988. IκB: a specific inhibitor of the NF-κB transcription factor. Science **242:**540–546.
5. **Baeuerle, P. A., and T. Henkel.** 1994. Function and activation of NF-κB in the immune system. Annu. Rev. Immunol. **12:**141–179.
6. **Bergelson, J. M., and R. W. Finberg.** 1993. Integrins as receptors for virus attachment and cell entry. Trends Microbiol. **1:**287–288.
7. **Berridge, M. J.** 1987. Inositol triphosphate and diacylglycerol: two interacting second messengers. Annu. Rev. Biochem. **56:**159–193.
8. **Boldogh, I., S. Abubakar, and T. Albrecht.** 1990. Activation of proto-oncogenes: an immediate early event in human cytomegalovirus. Science **247:**561–564.
9. **Boldogh, I., M. P. Fons, and T. Albrecht.** 1993. Increased levels of sequence-specific DNA-binding proteins in human cytomegalovirus-infected cells. Biochem. Biophys. Res. Commun. **197:**1505–1510.
10. **Britt, W. J., and D. Auger.** 1986. Synthesis and processing of the envelope gp55-116 complex of human cytomegalovirus. J. Virol. **58:**185–191.
11. **Chee, M. S., A. T. Bankier, S. Beck, R. Bohni, C. M. Brown, R. Cerny, T. Horsnell, C. A. Hutchison III, T. Kouzarides, J. A. Martignetti, E. Preddie, S. C. Satchwell, P. Tomlinson, K. M. Weston, and B. G. Barrell.** 1990. Analysis of the protein-coding content of the sequence of human cytomegalovirus strain AD169. Curr. Top. Microbiol. Immunol. **154:**125–169.
12. **Chen, L. I., T. Nishinaka, K. Kwan, I. Kitabayashi, K. Yokoyama, Y.-H. F. Fu, S. Grunwald, and R. Chiu.** 1994. The retinoblastoma gene product RB stimulates Sp1-mediated transcription by liberating Sp1 from a negative regulator. Mol. Cell. Biol. **14:**4380–4389.
13. **Cherrington, J. M., and E. S. Mocarski.** 1989. Human cytomegalovirus ie1 transactivates the α promoter-enhancer via an 18-base-pair repeat element. J. Virol. **63:**1435–1440.
14. **Cogswell, P. C., R. I. Scheinman, and A. S. Baldwin, Jr.** 1993. Promoter of the human NF-κB p50/p105 gene. Regulation by NF-κB subunits and by c-REL. J. Immunol. **150:**2794–2804.
15. **Compton, T.** 1995. Towards a definition of the HCMV entry pathway. Scand. J. Infect. Dis. **99**(Suppl.)**:**30–32.
16. **Compton, T., R. R. Nepomuceno, and D. M. Nowlin.** 1992. Human cytomegalovirus penetrates host cells by pH-independent fusion at the cell surface. Virology **191:**387–395.
17. **Compton, T., D. M. Nowlin, and N. R. Cooper.** 1993. Initiation of human cytomegalovirus infection requires initial interaction with cell surface heparan sulfate. Virology **193:**834–841.
18. **Cranage, M. P., T. Kouzarides, A. T. Bankier, S. Satchwell, K. Weston, P. Tomlinson, B. Barrell, H. Hart, S. E. Bell, A. C. Minson, and G. L. Smith.** 1986. Identification of the human cytomegalovirus glycoprotein B gene and induction of neutralizing antibodies via its expression in recombinant vaccinia virus. EMBO J. **5:**3057–3063.
19. **Cranage, M. P., G. L. Smith, S. E. Bell, H. Hart, C. Brown, A. T. Bankier, P. Tomlinson, B. G. Barrell, and T. G. Minson.** 1988. Identification and expression of a human cytomegalovirus glycoprotein with homology to the Epstein-Barr virus BXLF2 product, varicella-zoster virus gpIII, and herpes simplex virus type 1 glycoprotein H. J. Virol. **62:**1416–1422.
20. **Dalgliesh, A. G., P. C. L. Beverley, P. R. Clapham, D. H. Crawford, M. F. Greaves, and R. A. Weiss.** 1984. The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. Nature **312:**763–766.
21. **Dangoria, N. S., W. C. Breau, H. A. Anderson, D. M. Cishek, and L. C. Norkin.** 1996. Extracellular simian virus 40 induces an ERK/MAP kinase-independent signalling pathway that activates primary response genes and promotes virus entry. J. Gen. Virol. **77:**2173–2182.
22. **Demarchi, F., F. d'Adda di Fagagna, A. Falaschi, and M. Giacca.** 1996. Activation of transcription factor NF-κB by the Tat protein of human immunodeficiency virus type 1. J. Virol. **70:**4427–4437.
23. **Dynan, W. S., and D. Tjian.** 1983. The promoter-specific transcription factor Sp1 binds to upstream sequences in the SV40 early promoter. Cell **35:**79–87.
24. **Dynan, W. S., and R. Tjian.** 1983. Isolation of transcription factors that discriminate between different promoters recognized by RNA polymerase II. Cell **32:**669–680.
25. **Finco, T. S., and A. S. Baldwin, Jr.** 1995. Mechanistic aspects of NF-κB regulation: the emerging role of phosphorylation and proteolysis. Immunity **3:**263–272.
26. **Franzoso, G., L. Carlson, K. Brown, M. B. Daucher, P. Bressler, and U. Siebenlist.** 1996. Activation of the serum response factor by p65/NF-κB. EMBO J. **15:**3403–3412.
27. **Fujii, M., T. Minamino, M. Nomura, K. I. Miyamoto, J. Tanaka, and M. Seiki.** 1996. Selective activation of the proto-oncogene c-jun promoter by the transforming protein v-Rel. Oncogene **12:**2193–2202.
28. **Ghazal, P., and J. A. Nelson.** 1993. Transcription factors and viral regulatory proteins as potential mediators of human cytomegalovirus pathogenesis, p. 360–383. *In* Y. Becker, G. Darai, and E. S. Huang (ed.), Molecular aspects of human cytomegalovirus diseases. Springer-Verlag KG, Berlin, Germany.
29. **Gibson, W.** 1993. Molecular biology of human cytomegalovirus, p. 303–329. *In* Y. Becker, G. Darai, and E. S. Huang (ed.), Molecular aspects of human cytomegalovirus diseases. Springer-Verlag KG, Berlin, Germany.
30. **Haskill, S., A. A. Beg, S. M. Tompkins, J. S. Morris, A. D. Yurochko, A. Sampson-Johannes, K. Mondal, P. Ralph, and A. S. Baldwin, Jr.** 1991. Characterization of an immediate-early gene induced in adherent monocytes that encodes IκB-like activity. Cell **65:**1281–1289.
31. **Huang, E.-S.** 1975. Human cytomegalovirus. III. Virus-induced DNA polymerase. J. Virol. **16:**298–310.

32. **Huang, E.-S., S.-T. Chen, and J. S. Pagano.** 1973. Human cytomegalovirus. I. Purification and characterization of viral DNA. J. Virol. **12:**1473–1481.
33. **Huang, E.-S., and T. F. Kowalik.** 1993. The pathogenicity of human cytomegalovirus: an overview, p. 1–45. *In* Y. Becker, G. Darai, and E. S. Huang (ed.), Molecular aspects of human cytomegalovirus diseases. Springer-Verlag KG, Berlin, Germany.
34. **Hwang, E. S., J. Y. Seoh, C. G. Park, Y. H. Kook, and C. Y. Cha.** 1992. Characterization of monoclonal antibodies against human cytomegalovirus (HCMV) glycoproteins. J. Korean Soc. Microbiol. **27:**545–553.
35. **Jahn, G., and M. Mach.** 1990. Human cytomegalovirus phosphoproteins and glycoproteins and their coding regions. Curr. Top. Microbiol. Immunol. **154:**171–185.
36. **Kadonaga, J. T., K. R. Carner, F. R. Masiarz, and R. Tjian.** 1987. Isolation of cDNA encoding transcription factor Sp1 and functional analysis of the DNA binding domain. Cell **51:**1079–1090.
37. **Kaye, J. F., U. A. Compel, and A. C. Minson.** 1992. Glycoprotein H of human cytomegalovirus (HCMV) forms a stable complex with HCMV UL115 gene product. J. Gen. Virol. **73:**2693–2698.
38. **Keay, S., and B. Baldwin.** 1991. Anti-idiotypic antibodies that mimic gp86 of human cytomegalovirus inhibit viral fusion but not attachment. J. Virol. **65:**5124–5128.
39. **Keay, S., and B. Baldwin.** 1992. The human fibroblast receptor for gp86 of human cytomegalovirus is a phosphorylated glycoprotein. J. Virol. **66:**4834–4838.
40. **Keay, S., B. Baldwin, M. W. Smith, S. S. Wasserman, and W. F. Goldman.** 1995. Increases in $[Ca^{2+}]_i$ mediated by the 92.5-kDa putative cell membrane receptor for HCMV gp86. Am. J. Physiol. **269:**C11–C21.
41. **Keay, S., and B. R. Baldwin.** 1996. Evidence for the role of cell phosphorylation in human cytomegalovirus/host cell fusion. J. Gen. Virol. **77:**2597–2604.
42. **Keay, S., T. C. Merigan, and L. Rasmussen.** 1989. Identification of cell surface receptors for the 86-kilodalton glycoprotein of human cytomegalovirus. Proc. Natl. Acad. Sci. USA **86:**10100–10103.
43. **Keay, S., L. Rasmussen, and L. Merigan.** 1988. Syngeneic monoclonal anti-idiotypic antibodies that bear the internal image of a human cytomegalovirus neutralization epitope. J. Immunol. **140:**944–948.
44. **Kerry, J. A., M. A. Priddy, T. L. Staley, T. R. Jones, and R. M. Stenberg.** 1997. The role of ATF in regulating human cytomegalovirus DNA polymerase (UL54) promoter during viral infection. J. Virol. **71:**2120–2126.
45. **Kessler, D. J., M. P. Duyao, D. B. Spicer, and G. E. Sonenshein.** 1992. NF-κB-like factors mediate interleukin 1 induction of *c-myc* gene transcription in fibroblasts. J. Exp. Med. **176:**787–792.
46. **Kim, S.-J., U. S. Onwuta, Y. I. Lee, R. Li, M. R. Botchan, and P. D. Robbins.** 1992. The retinoblastoma gene product regulates Sp1-mediated transcription. Mol. Cell. Biol. **12:**2455–2463.
47. **Kingsley, C., and A. Winoto.** 1992. Cloning of GT box-binding proteins: a novel Sp1 multigene family regulating T-cell receptor gene expression. Mol. Cell. Biol. **12:**4251–4261.
48. **Klatzmann, D., E. Champagne, S. Chamaret, J. Gruest, D. Guetard, T. Hercend, J.-C. Gluckman, and L. Montagnier.** 1984. T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV. Nature **312:**767–768.
49. **Kowalik, T. F., B. Wing, J. S. Haskill, J. C. Azizkhan, A. S. Baldwin, Jr., and E.-S. Huang.** 1993. Multiple mechanisms are implicated in the regulation of NF-κB activity during human cytomegalovirus infection. Proc. Natl. Acad. Sci. USA **90:**1107–1111.
50. **Lindholm, P. F., M. Tamami, J. Makowski, and J. N. Brady.** 1996. Human T-cell lymphotropic virus type 1 Tax_1 activation of NF-κB: involvement of the protein kinase C pathway. J. Virol. **70:**2525–2532.
51. **Majello, B., P. De Luca, G. Hagen, G. Suske, and L. Lania.** 1994. Different members of the Sp1 multigene family exert opposite transcriptional regulation of the long terminal repeat of HIV-1. Nucleic Acids Res. **22:**4914–4921.
52. **Majello, B., P. De Luca, G. Suske, and L. Lania.** 1995. Differential transcriptional regulation of *c-myc* promoter through the same DNA binding sites targeted by Sp1-like proteins. Oncogene **10:**1841–1848.
53. **Montgomery, R. I., M. S. Warner, B. J. Lum, and P. G. Spear.** 1996. Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. Cell **87:**427–436.
54. **Navarro, D., P. Paz, S. Tugizov, K. Topp, J. La Vail, and L. Pereira.** 1993. Glycoprotein B of human cytomegalovirus promotes virion penetration into cells, transmission of infection from cell to cell, and fusion of infected cells. Virology **197:**143–158.
55. **Nowlin, D. M., N. R. Cooper, and T. Compton.** 1991. Expression of a human cytomegalovirus receptor correlates with infectibility of cells. J. Virol. **65:**3114–3121.
56. **Otto, M., A. Gunther, H. Fan, O. Rick, and R. T. Huang.** 1994. Identification of annexin 33 kDa in cultured cells as a binding protein of influenza virus. FEBS Lett. **356:**125–129.
57. **Pachl, C., W. S. Probert, K. M. Hermsen, F. R. Masiarz, L. Rasmussen, T. C. Merigan, and R. R. Spaete.** 1989. The human cytomegalovirus strain Towne glycoprotein H encodes glycoprotein p86. Virology **169:**418–426.
58. **Pereira, L.** 1994. Function of glycoprotein B homologues of the family Herpesviridae. Infect. Agents Dis. **3:**9–28.
59. **Qadri, I., D. Navarro, P. Paz, and L. Pereira.** 1992. Assembly of conformation-dependent neutralizing domains on glycoprotein B of human cytomegalovirus. J. Gen. Virol. **73:**2913–2921.
60. **Rasmussen, L., J. Mullenax, R. Nelson, and T. C. Merigan.** 1985. Viral polypeptides detected by a complement-dependent neutralizing murine monoclonal antibody to human cytomegalovirus. J. Virol. **55:**274–280.
61. **Rasmussen, L. E., R. M. Nelson, D. C. Kelsall, and T. C. Merigan.** 1984. Murine monoclonal antibody to a single protein neutralizes the infectivity of human cytomegalovirus. Proc. Natl. Acad. Sci. USA **81:**876–880.
62. **Sambucetti, L. C., J. M. Cherrington, G. W. Wilkinson, and E. S. Mocarski.** 1989. NF-κB activation of the cytomegalovirus enhancer is mediated by a viral transactivator and by T cell stimulation. EMBO J. **8:**4251–4258.
63. **Scheinman, R. I., A. A. Beg, and A. S. Baldwin, Jr.** 1993. NF-κB p100 (Lyt-10) is a component of H2TF1 and can function as an IκB-like molecule. Mol. Cell. Biol. **13:**6089–6101.
64. **Siebenlist, U., G. Franzoso, and K. Brown.** 1994. Structure, regulation and function of NF-κB. Annu. Rev. Cell Biol. **10:**405–455.
65. **Sinclair, A. J., and P. J. Farrell.** 1995. Host cell requirements for efficient infection of quiescent primary B lymphocytes by Epstein-Barr virus. J. Virol. **69:**5461–5468.
66. **Stenberg, R. M.** 1993. Immediate-early genes of human cytomegalovirus: organization and function, p. 330–359. *In* Y. Becker, G. Darai, and E. S. Huang (ed.), Molecular aspects of human cytomegalovirus diseases. Springer-Verlag KG, Berlin, Germany.
67. **Su, F., and R. J. Schneider.** 1996. Hepatitis B virus HBx protein activates transcription factor NF-κB by acting on multiple cytoplasmic inhibitors of *rel*-related proteins. J. Virol. **70:**4558–4566.
68. **Tanner, J. E., C. Alfieri, T. A. Chatila, and F. Diaz-Mitoma.** 1996. Induction of interleukin-6 after stimulation of human B-cell CD21 by Epstein-Barr virus glycoproteins gp350 and gp220. J. Virol. **70:**570–575.
69. **Thanos, D., and T. Maniatis.** 1995. NF-κB: a lesson in family values. Cell **80:**529–532.
70. **Udvadia, A. J., K. T. Rogers, P. D. R. Higgins, Y. Murata, K. H. Martin, P. A. Humphrey, and J. M. Horowitz.** 1993. Sp-1 binds promoter elements regulated by the RB protein and Sp-1-mediated transcription is stimulated by RB coexpression. Proc. Natl. Acad. Sci. USA **90:**3265–3269.
71. **Udvania, A. J., D. J. Templeton, and J. M. Horowitz.** 1995. Functional interactions between the retinoblastoma (Rb) protein and Sp-family members: superactivation by Rb requires amino acids necessary for growth suppression. Proc. Natl. Acad. Sci. USA **92:**3953–3957.
72. **Wickham, T. J., P. Mathias, D. A. Cheresh, and G. R. Nemerow.** 1993. Integrins αVβ3 and αVβ5 promote adenovirus internalization but not virus attachment. Cell **73:**309–319.
73. **Wing, B. A., and E.-S. Huang.** Unpublished data.
74. **Wing, B. A., G. C. Y. Lee, and E.-S. Huang.** 1996. The human cytomegalovirus UL94 open reading frame encodes a conserved herpesvirus capsid/tegument-associated virion protein that is expressed with true late kinetics. J. Virol. **70:**3339–3345.
75. **Wright, J. F., A. Kurosky, and S. Wasi.** 1994. An endothelial cell-surface form of annexin II binds human cytomegalovirus. Biochem. Biophys. Res. Commun. **198:**983–989.
76. **Yurochko, A. D.** 1997. Adhesion molecules as signal transduction molecules, p. 155–180. *In* L. C. Paul and T. Issekutz (ed.), Targeting cell adhesion molecules for therapeutic application. Marcel Dekker, Inc., New York, N.Y.
77. **Yurochko, A. D., and E.-S. Huang.** Unpublished results.
78. **Yurochko, A. D., T. F. Kowalik, S.-M. Huong, and E.-S. Huang.** 1995. Human cytomegalovirus upregulates NF-κB activity by transactivating the NF-κB p105/p50 and p65 promoters. J. Virol. **69:**5391–5400.
79. **Yurochko, A. D., M. W. Mayo, E. E. Poma, A. S. Baldwin, Jr., and E.-S. Huang.** 1997. Induction of the transcription factor Sp1 during human cytomegalovirus infection mediates upregulation of the p65 and p105/p50 NF-κB promoters. J. Virol. **71:**4638–4648.